

Molecular typing and sequencing of adenovirus isolated from a conjunctivitis outbreak in a neonatal intensive care unit by PCR

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Aim: We aimed to evaluate the molecular typing of adenovirus isolated during an epidemic at the Ege University Children's Hospital neonatal intensive care unit (NICU).

Materials and methods: During the NICU outbreak management, 40 clinical samples (from 15 newborn infants and 25 health care providers) were sent to a microbiology laboratory in viral transport media. All the samples were processed using a direct fluorescent antibody (DFA) test and a shell vial cell culture followed by adenovirus polymerase chain reaction (PCR) and DNA sequencing. PCR and DNA sequencing for adenovirus hexon gene hypervariable regions 1–6 were done after DNA extraction from clinical specimens. Adenovirus typing was done using BLAST analysis.

Results: Ten adenoviruses were isolated from 4 out of 10 infants, 3 out of 5 hospital staff with conjunctivitis, and 3 asymptomatic staff. Ten positive samples were identified as adenovirus type 8 by using BLAST analysis.

Conclusion: We isolated adenovirus type 8, one of the most common serotypes causing conjunctivitis, during an adenovirus outbreak in our NICU. The highest positivity was obtained using the PCR method. Although DFA was positive in a limited number of cases, this test was applied rapidly at the beginning of the epidemic and contributed to the prevention of further spread.

Key words: Adenovirus, neonatal, outbreak

Introduction

Adenoviruses, divided into several serotypes, belong to the genus *Mastadenovirus* of the family *Adenoviridae*, and cause widespread infections in different age groups all around the world. The different serotypes can lead to a wide variety of clinical manifestations such as conjunctivitis, keratoconjunctivitis, upper and lower respiratory tract infections, and hemorrhagic cystitis (1,2).

Serotypes 2, 3, 4, 6, 7, 8, 19, and 37 are associated with conjunctivitis and keratoconjunctivitis. Adenoviral infections are common in patients with immune deficiency secondary to T or B cell

dysfunction, and are seen in the normal population as well. In addition, newborn infants, particularly premature infants, are very susceptible to adenoviral infections due to a decreased antibody production capacity of B lymphocytes and a lack of maternal antibodies (2,3). In this study, diagnostic tests were intended to be implemented using the direct fluorescent antibody (DFA) test and cell culture method in the first stage of an adenovirus outbreak in a neonatal intensive care unit (NICU). Polymerase chain reaction (PCR) and DNA sequencing methods were applied to the specimens stored at –80 °C for adenovirus typing later on.

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Materials and methods

Subject populations and sample types

During an adenovirus outbreak at the NICU of the Ege University Faculty of Medicine between 14 September and 17 October 2009, 10 of 15 newborns (6 male, 9 female) and 5 of 25 hospital staff (4 male, 21 female) had symptoms of acute conjunctivitis. Considering the possibility of an outbreak, subconjunctival and nasopharyngeal swab samples were collected from all 40 individuals and sent to the laboratory in viral transport medium (Universal Transport Medium Kit, Copan Diagnostics, Italy). During the epidemic, a DFA test and a shell vial cell culture were performed on all samples. Samples were stored at -80°C to be submitted for PCR and DNA sequencing.

DFA test and shell vial cell culture

DFA tests and shell vial cell cultures were performed on all the specimens at the same time. Specimens were mixed and centrifuged at 1000 rpm at 4°C for 10 min. The supernatant was used for the shell vial cell culture. The cell pellet was resuspended in phosphate-buffered saline. The cell suspension (100 μL) was placed in a cytofunnel and cytocentrifuged at 2000 rpm for 5 min. The slides were then air-dried and fixed in prechilled acetone for 10 min at -20°C . The cytocentrifuged specimens were stained with a FITC-labeled monoclonal antibody specific for adenovirus (Adenovirus Kit, Light Diagnostics, Millipore, USA) according to the manufacturer's protocol. The presence of at least 3 cells with typical staining was considered to be positive.

One shell vial containing human laryngeal carcinoma (HEp-2, German Collection of Microorganisms and Cell Cultures, DSMZ, Germany) was prepared for each specimen. Each vial was inoculated with 0.2 mL of specimen supernatant for the recovery of the adenovirus. The vials were centrifuged at $700 \times g$ for 1 h at 25°C and incubated at 37°C for 1 h. Supernatants were aspirated from each vial. Subsequently, 1 mL of isolation medium containing Eagle's MEM supplemented with 10% fetal calf serum and antibiotics (BiochromAG, Germany) was added to the vials. The vials were then incubated in a moist chamber at 37°C in a 5% CO_2 atmosphere for 48 h as previously described (4). Cover slips were fixed in prechilled acetone for 10 min at -20°C and stained with a FITC-labeled monoclonal antibody

specific for adenovirus (Light Diagnostic, Millipore USA) according to the manufacturer's protocol. The cover slips that had one or more fluorescing inclusions were considered to be positive.

PCR and DNA sequencing

DNA extractions were performed from 200 μL of clinical specimen in viral transport medium using the QIAGEN MinElute Virus Spin Kit (QIAGEN GmbH, Germany) according to the manufacturer's protocol. The PCR primers Adhex F1 (nt 19135–19160; 5'-TICTTTGACATTCGIGGIGTICTIGA-3') and Adhex R1 (nt 20009–20030; 5'-CTGTTCIACIGCCTGRTTCCACA-3'), which were used for amplification, were from those previously described by Lu and Erdman (5). If insufficient DNA for sequencing was amplified from the first reaction, a nested PCR was performed using primers Adhex F2 (nt 19165–19187; 5'-GGYCCYAGYTTYAARCCCTAYTC-3') and Adhex R2 (nt 19960–19985; 5'-GGTTCGTGTCICCCAGAGARTCIAGCA-3'). Briefly, PCR amplification was performed using 25- μL reaction volumes containing 12.5 μL of SYBR super mixture (DYEnamic ET Terminator Cycle Sequencing Kit, UK), 2.5 μL of Adhex F1 primer, 2.5 μL of Adhex R1 primer, 5 μL of nucleic acid extract, and 2.5 μL of dH_2O at the following settings: 95°C for 15 min of denaturation followed by 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 5 min. The amplified products were separated on 1% agarose gels for determination of concentration and purified with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Germany). Sequencing was performed using the amplification primers and the Sequence Reagent Mix DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., USA) on an ABI PRISM 310 Genetic Analyzer. Briefly, PCR amplification was performed using 10- μL reaction volumes containing 0.6 μL of primers, 3 μL of sequence reagent mix, and a maximum of 6.4 μL of specimen (according to specimen density) at the following settings: 95°C for 20 s of denaturation followed by 35 cycles of 50°C for 25 s and 60°C for 2 min, with a final extension of 4°C for 10 min. The amplified products were purified with sodium acetate and EtOH. After the reaction was over, 1 μL of 1.5 M sodium acetate, 10 μL of dH_2O , and 80 μL of 100% ethanol were added to each tube and mixed

for 10 s. The tubes were centrifuged at 12,500 rpm for 15 min at 20 °C. The liquid phase was discarded and 200 mL of 70% ethanol was added to the bottom of the collapsed portion and centrifuged at 12,500 rpm for 3 min at 20 °C. The liquid phase was discarded and the bottom part that collapsed was dried in a thermomixer device at 56 °C for 10 min. Finally, 15 µL of template suppression reagent was added to the supernatant. The tubes were loaded into an ABI PRISM 310 Genetic Analyzer after 10 s of mixing and spin centrifugation. The adenovirus sequences obtained were typed using BLAST analysis with a nucleotide database (GenBank+EMBL+DDBJ+PDB sequences) (6).

Results

During the epidemic in our NICU, 10 of the 40 subjects (25%) were found to be adenovirus-positive. An adenovirus was identified in a total of 10 subjects, 7 symptomatic and 3 asymptomatic. Both nasopharyngeal and conjunctival swabs were positive among the 7 symptomatic patients. The symptomatic group consisted of 4 infants and 3 hospital staff with conjunctivitis. Of the symptomatic patients with conjunctivitis, 6 infants and 2 hospital staff were adenovirus-negative. On the other hand, the nasopharyngeal swabs of 3 asymptomatic hospital staff were detected as adenovirus-positive. Conjunctival swabs of these 3 patients were adenovirus-negative. All samples (nasopharyngeal and conjunctival) from the other 5 asymptomatic infants and the 17 asymptomatic hospital staff were adenovirus-negative (Table 1).

Four of the positive specimens were positive based on the DFA, cell culture, and PCR tests; 3 specimens

were positive based on the cell culture and PCR tests; and the remaining 3 samples were positive only by PCR. Thirty samples were negative by all 3 methods (Table 2). Ten adenovirus strains were typed as adenovirus type 8 by BLAST analysis (maximum identification: 99%).

Discussion

Sporadic outbreaks of adenoviral conjunctivitis generally occur with the spread of pediatric conjunctivitis infections to other individuals. In addition, sensitive individuals might be infected due to asymptomatic adenovirus circulation in the community. Infected people can easily infect others through hand contact due to the fact that viral shedding starts about 2 weeks before the onset of clinical signs. Nosocomial infections often occur due to spread of the virus through adenovirus-infected hands or equipment used for examination in ophthalmology clinics. Adenoviruses are more resistant to antiseptics than other viruses. Therefore, the equipment and towels that are used during examinations in ophthalmology clinics are more likely to be contaminated with the virus. This may cause sporadic outbreaks among patients examined in the ophthalmology clinics, hospital staff, and other patients (7,8). After the research for this study was conducted, the onset of the epidemic was reported to have started 3 days after the routine examination of 5 NICU patients for retinopathy of prematurity. Conjunctivitis was also reported in the ophthalmological examinations of these infants 3 days later (9).

The most common types of adenoviruses causing ocular infections are types 4, 8, 19, and

Table 1. Distribution of adenoviruses in newborns and health care personnel according to sample type.

	Positive nasopharyngeal swab	Positive conjunctival swab	%
Symptomatic newborn (n = 10)	4	4*	
Symptomatic health care personnel (n = 5)	3	3*	
Total symptomatic patients (n = 15)	7	7*	46.7
Asymptomatic newborn (n = 5)	0	0	
Asymptomatic health care personnel (n = 20)	3	0	
Total asymptomatic persons (n = 25)	3	0	12.0

*The same patient had positive conjunctival and nasopharyngeal swab samples for adenovirus.

Table 2. Distribution of positive DFA, shell vial cell culture, and PCR assay results in clinical specimens.

	Positive results by assay			
	DFA+CC+PCR*	CC+PCR	PCR	Total
Symptomatic newborn (n = 10)	3	–	1	4
Symptomatic health care personnel (n = 5)	1	–	2	3
Asymptomatic newborn (n = 5)	–	–	–	–
Asymptomatic health care personnel (n = 20)	–	3	–	3

*DFA = direct fluorescent antibody test, CC = cell culture, and PCR = polymerase chain reaction.

37. Acute conjunctivitis is often found with pharyngoconjunctivitis syndrome (2,10). In a study conducted in Turkey, the most common type of adenovirus found in patients with acute conjunctivitis was type 8 (11). In this study, although they were not clinically diagnosed with pharyngoconjunctivitis, 7 patients with conjunctivitis had adenovirus type 8. The nasopharyngeal swab samples of these 7 patients were also positive for adenovirus. Additionally, adenovirus was not detected by any of the 3 methods in 8 of the 15 patients with conjunctivitis. This may be due to a lack of good quality samples (the conjunctiva is a difficult type of epithelial tissue to sample), unsuitable transport conditions, or partial reduction in the quantity of viruses during the process of freeze thawing. At the beginning of this epidemic, samples were prepared for DFA testing as soon as they arrived at the laboratory and were evaluated on slides on the same day. The remaining samples were divided into 2 aliquots, 1 of which was used for the shell vial cell culture within an average of 3 days, and the other was stored at -80°C for about 6 months to be used in PCR and sequencing tests. In this study, the maximum positivity ratio was achieved using the PCR test; however, the loss of viral load during this period may have had an adverse effect on the PCR results. With the use of the DFA, PCR, and cell culture methods, adenovirus was identified in 46.7% (7/15) of 10 newborns and 5 hospital staff with symptoms of conjunctivitis. During the outbreak, DFA and cell culture tests were applied to samples from asymptomatic newborns and hospital staff. Only 3 hospital staff had positive nasopharyngeal

adenovirus swab samples. Nasopharyngeal swab samples of these individuals were also subsequently found to be positive by the PCR test.

DFA, cell culture, and PCR methods are widely used in the laboratory diagnosis of adenoviruses. The serum neutralization and hemagglutination inhibition tests are the classic methods used for typing of adenoviruses (5). These tests are labor-intensive methods requiring hyperimmune polyclonal antibodies and give results in a few weeks. Furthermore, evaluation of the test results can be difficult due to some cross-reactivity between serotypes of adenovirus (12). Recently, it has been reported that the results from PCR and DNA sequencing studies with part of a hexon gene were more practical and were found to correlate with the results from studies done with classic methods and routine molecular typing (1,2). In this study, adenovirus typing was done using PCR and DNA sequencing of hypervariable regions 1–6 of the hexon gene and BLAST analysis of DNA sequences.

In conclusion, nosocomial infections caused by viruses and bacteria are still important issues (13–15). During the epidemic in our NICU, adenovirus type 8, one of the most common serotypes causing conjunctivitis, was identified. Although a limited number of patients were positive based on the shell vial cell culture and DFA tests, adenovirus positivity was detected among asymptomatic hospital staff within 3 days and they were immediately removed from the NICU. DFA testing may be applied to clinical samples at the beginning of an epidemic to prevent the spread of epidemics.

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